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(54) Title: PROCESS FOR DIRECT SEQUENCING DURING TEMPLATE AMPLIFICATION			
(57) Abstract Processes and kits for simultaneously amplifying and sequencing nucleic acid molecules, and performing high throughput DNA sequencing are described.			

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PROCESS FOR DIRECT SEQUENCING DURING TEMPLATE AMPLIFICATION

5 Background of the Invention*DNA Sequencing*

Current knowledge regarding gene structure, the control of gene activity and the function of cells on a molecular level all arose based on the determination of the
10 base sequence of millions of DNA molecules. DNA sequencing is still critically important in research and for genetic therapies and diagnostics, (e.g., to verify recombinant clones and mutations).

DNA, a polymer of deoxyribonucleotides, is found in all living cells and
15 some viruses. DNA is the carrier of genetic information, which is passed from one generation to the next by homologous replication of the DNA molecule. Information for the synthesis of all proteins is encoded in the sequence of bases in the DNA.

To obtain the genetic information and therefore to reveal the base
20 sequence of a given DNA molecule, chemical and enzymatic sequencing methods have been developed. DNA-sequencing as proposed by Maxam-Gilbert (Maxam, A.M., W. Gilbert, Proc. Natl. Acad. Sci. USA, 74:560-564 1977) is a chemical method of determining base composition of a nucleic acid molecule. A single stranded DNA molecule with radioactive label at its 5' end is chemically modified in four base specific
25 reactions and then cleaved at the modified positions. The cleavage products are separated on a polyacrylamide gel and typically are detected by autoradiography.

Currently favored is the enzymatic chain termination reaction according to the Sanger-sequencing method (Sanger, F. et. al., Proc. Natl. Acad. Sci. USA, 74:5463-
30 5467 1977). In the Sanger method, the four base specific sets of DNA fragments are formed by starting with a primer/template system elongating the primer into the unknown DNA sequence area and thereby copying the template and synthesizing complementary strands using a DNA polymerase in the presence of chain-terminating reagents. The

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chain-terminating event is achieved by incorporating into the four separate reaction mixtures in addition to the four normal deoxynucleoside triphosphates, dATP, dGTP, dTTP and dCTP, only one of the chain-terminating dideoxynucleoside triphosphates, ddATP, ddGTP, ddTTP or ddCTP, respectively, in a limiting small concentration. The
5 incorporation of a ddNTP lacking the 3' hydroxyl function into the growing DNA strand by the enzyme DNA polymerase leads to chain termination through preventing the formation of a 3'-5'-phosphodiester bond by DNA polymerase. Due to the random incorporation of the ddNTPs, each reaction leads to a population of base specific terminated fragments of different lengths, which all together represent the sequenced
10 DNA-molecule.

A recent modification of the Sanger sequencing strategy involves the degradation of phosphorothioate-containing DNA fragments obtained by using alpha-thio dNTP instead of the normally used ddNTPs during the primer extension reaction
15 mediated by DNA polymerase (Labeit *et al.*, *DNA* 5, 173-177 (1986); Amersham, PCT-Application GB86/00349; Eckstein *et al.*, *Nucleic Acids Res.* 16, 9947 (1988)). Here, the four sets of base-specific sequencing ladders are obtained by limited digestion with exonuclease III or snake venom phosphodiesterase, subsequent separation on PAGE and visualization by radioisotopic labeling of either the primer or
20 one of the dNTPs. In a further modification, the base-specific cleavage is achieved by alkylating the sulphur atom in the modified phosphodiester bond followed by a heat treatment (Max-Planck-Gesellschaft, DE 3930312 A1).

DNA Amplification

25 DNA can be amplified by a variety of procedures including cloning (Sambrook et al., *Molecular Cloning : A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989), polymerase chain reaction (PCR) (C.R. Newton and A. Graham, PCR, BIOS Publishers, 1994), ligase chain reaction (LCR) (F. Barany *Proc. Natl. Acad. Sci USA* 88, 189-93 (1991), strand displacement amplification (SDA) (G. Terrance Walker et al., *Nucleic Acids Res.* 22, 2670-77 (1994)) and variations such as
30 RT-PCR, allele-specific amplification (ASA) etc.

The polymerase chain reaction (Mullis, K. et al., *Methods Enzymol.*, 155:335-350 1987) permits the selective in vitro amplification of a particular DNA region by mimicking the phenomena of in vivo DNA replication. Required reaction components are single stranded DNA, primers (oligonucleotide sequences complementary to the 5' and 3' ends of a defined sequence of the DNA template), deoxynucleotidetriphosphates and a DNA polymerase enzyme. Typically, the single stranded DNA is generated by heat denaturation of provided double strand DNA. The reaction buffers contain magnesium ions and co-solvents for optimum enzyme stability and activity.

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The amplification results from a repetition of such cycles in the following manner: The two different primers, which bind selectively each to one of the complementary strands, are extended in the first cycle of amplification. Each newly synthesized DNA then contains a binding site for the other primer. Therefore each new DNA strand becomes a template for any further cycle of amplification enlarging the template pool from cycle to cycle. Repeated cycles theoretically lead to exponential synthesis of a DNA-fragment with a length defined by the 5' termini of the primer.

15

Initial PCR experiments used thermolabile DNA polymerase. However, thermolabile DNA polymerase must be continually added to the reaction mixture after each denaturation cycle. Major advances in PCR practice were the development of a polymerase, which is stable at the near-boiling temperature (Saiki, R. et al., *Science* 239:487-491 1988) and the development of automated thermal cyclers.

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The discovery of thermostable polymerases also allowed modification of the Sanger sequencing reaction with significant advantages. The polymerization reaction could be carried out at high temperature with the use of thermostable DNA polymerase in a cyclic manner (cycle sequencing). The conditions of the cycles are similar to those of the PCR technique and comprise denaturation, annealing, and extension steps. Depending on the length of the primers only one annealing step at the beginning of the reaction may be sufficient. Carrying out a sequencing reaction at

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high temperature in a cyclic manner provides the advantage that each DNA strand can serve as template in every new cycle of extension which reduces the amount of DNA necessary for sequencing, thereby providing access to minimal volumes of DNA, as well as resulting in improved specificity of primer hybridisation at higher temperature and the reduction of secondary structures of the template strand.

However, amplification of the terminated fragments is linear in conventional cycle sequencing approaches. A recently developed method, called semi-exponential cycle sequencing shortens the time required and increases the extent of amplification obtained from conventional cycle sequencing by using a second reverse primer in the sequencing reaction. However, the reverse primer only generates additional template strands if it avoids being terminated prior to reaching the sequencing primer binding site. Needless to say, terminated fragments generated by the reverse primer can not serve as a sufficient template. Therefore, in practice, amplification by the semi-exponential approach is not entirely exponential. (Sarkat, G. and Bolander Mark E., Semi Exponential Cycle Sequencing Nucleic Acids Research, 1995, Vol. 23, No. 7, p. 1269-1270).

As pointed out above, current nucleic acid sequencing methods require relatively large amounts (typically about 1 g) of highly purified DNA template. Often, however, only a small amount of template DNA is available. Although amplifications may be performed, amplification procedures are typically time consuming, can be limited in the amount of amplified template produced and the amplified DNA must be purified prior to sequencing. A streamlined process for amplifying and sequencing DNA is needed, particularly to facilitate highthroughput nucleic acid sequencing.

Summary of the Invention

In general, the instant invention provides a one-step process for generating from a DNA template, base specifically terminated fragments of sufficient quantity to enable DNA sequencing.

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According to the process of the invention, a combined amplification and termination reaction is performed using at least two different polymerase enzymes, each having a different affinity for the chain terminating nucleotide, so that polymerization by
5 an enzyme with relatively low affinity for the chain terminating nucleotide leads to exponential amplification whereas an enzyme with relatively high affinity for the chain terminating nucleotide terminates the polymerization and yields sequencing products.

In another aspect, the invention features kits for directly amplifying
10 nucleic acid templates and generating base specifically terminated fragments. In one embodiment, the kit can comprise an appropriate amount of: i) a complete set of chain-elongating nucleotides; ii) at least one chain-terminating nucleotide; (iii) a first DNA polymerase, which has a relatively low affinity towards the chain terminating nucleotide; and (iv) a second DNA polymerase, which has a relatively high affinity towards the chain
15 terminating nucleotide. The kit can also optionally include an appropriate primer or primers, appropriate buffers as well as instructions for use.

The instant invention allows DNA amplification and termination to be performed in one reaction vessel. Due to the use of two polymerases with different
20 affinities for dideoxy nucleotide triphosphates, exponential amplification of the target sequence can be accomplished in combination with a termination reaction nucleotide. In addition, the process obviates the purification procedures, which are required when amplification is performed separately from base terminated fragment generation. Further, the instant process requires less time to accomplish than separate amplification and base
25 specific termination reactions.

When combined with a detection means, the process can be used to detect and/ or quantitate a particular nucleic acid sequence where only small amounts of template are available and fast and accurate sequence data acquisition is desirable. For
30 example, when combined with a detection means, the process is useful for sequencing unknown genes or other nucleic acid sequences and for diagnosing or monitoring certain

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diseases or conditions, such as genetic diseases, chromosomal abnormalities, genetic predispositions to certain diseases (e.g. cancer, obesity, arteriosclerosis) and pathogenic (e.g. bacterial, viral, fungal, protistal) infections. Further, when double stranded DNA molecules are used as the starting material, the instant process provides an opportunity to simultaneously sequence both strands, thereby providing greater certainty of the sequence data obtained or acquiring sequence information from both ends of a longer template.

The above and further features and advantages of the instant invention will become clearer from the following Detailed Description and Claims.

Brief Description of the Figures

FIGURE 1 shows sequence data from an ABI-Prism automated sequencer (Model 373A) using a p0M8 derived recombinant plasmid with a 400 base pair (BP) insert from the *rrnB* gene of *E. coli* as the template in the reaction described in the following Example 1. The figure shows a reliable sequence readable to about 440 BP which is the length of the PCR product.

FIGURE 2 shows sequence data from an ABI-Prism automated sequencer (Model 373A) again using the p0M8 derived recombinant plasmid with a 400 BP insert of from the *rrnB* gene of *E. coli*. However, because the sequencing reaction was carried out using standard sequencing protocols on a small amount of template (50ng), no reliable sequence was obtained.

FIGURE 3 is a schematic of a combined amplification and sequencing reaction using two polymerases and dye-labeled dideoxynucleotide triphosphate for detection and two reverse oriented primers.

FIGURE 4 is a fluorescent chromatogram plot of a forward sequence ladder generated in a simultaneous amplification and sequencing reaction.

FIGURE 5 is a fluorescent chromatogram plot of a reverse sequence ladder generated in a simultaneous amplification and sequencing reaction.

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Detailed Description of the Invention

In general, the invention features a process for directly amplifying and base specifically terminating a nucleic acid molecule. According to the process of the invention, a combined amplification and termination reaction is performed on a nucleic acid template using: i) a complete set of chain-elongating nucleotides; ii) at least one chain-terminating nucleotide; and (iii) a first DNA polymerase, which has a relatively low affinity towards the chain terminating nucleotide; and (iv) a second DNA polymerase, which has a relatively high affinity towards the chain terminating nucleotide, so that polymerization by the enzyme with relatively low affinity for the chain terminating nucleotide leads to amplification of the template, whereas the enzyme with relatively high affinity for the chain terminating nucleotide terminates the polymerization and yields sequencing products.

The combined amplification and sequencing can be based on any amplification procedure that employs an enzyme with polynucleotide synthetic ability (e.g. polymerase). One preferred process, based on the polymerase chain reaction (PCR), is comprised of the following three thermal steps: 1) denaturing a double stranded (ds) DNA molecule at an appropriate temperature and for an appropriate period of time to obtain the two single stranded (ss) DNA molecules (the template: sense and antisense strand); 2) contacting the template with at least one primer that hybridizes to at least one ss DNA template at an appropriate temperature and for an appropriate period of time to obtain a primer containing ss DNA template; 3) contacting the primer containing template at an appropriate temperature and for an appropriate period of time with: (i) a complete set of chain elongating nucleotides, (ii) at least one chain terminating nucleotide, (iii) a first DNA polymerase, which has a relatively low affinity towards the chain terminating nucleotide; and (iv) a second DNA polymerase, which has a relatively high affinity towards the chain terminating nucleotide.

Steps 1)- 3) can be sequentially performed for an appropriate number of times (cycles) to obtain the desired amount of amplified sequencing ladders. The

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quantity of the base specifically terminated fragment desired dictates how many cycles are performed. Although an increased number of cycles results in an increased level of amplification, it may also detract from the sensitivity of a subsequent detection. It is therefore generally undesirable to perform more than about 50 cycles, and is more
5 preferable to perform less than about 40 cycles (e.g. about 20-30 cycles).

In a preferred embodiment, the first denaturation step is performed at a temperature in the range of about 85°C to about 100°C (most preferably about 92°C to about 96°C) for about 20 seconds (s) to about 2 minutes (most preferably about 30s- 1
10 minute). The second hybridization step is preferably performed at a temperature, which is in the range of about 40°C to about 80°C (most preferably about 45°C to about 72°C) for about 20s to about 2 minutes (most preferably about 30s- 1 minute). The third, primer extension step is preferably performed at about 65°C to about 80°C (most preferably about 70°C to about 74°C) for about 30 s to about 3 minutes (most preferably
15 about 1 to about 2 minutes).

In order to obtain sequence information on both the sense and antisense strands of a DNA molecule simultaneously, each of the single stranded sense and antisense templates generated from the denaturing step can be contacted with appropriate
20 primers in step 2), so that amplified and chain terminated nucleic acid molecules generated in step 3), are complementary to both strands.

Another preferred process for simultaneously amplifying and chain terminating a nucleic acid sequence is based on strand displacement amplification (SDA)
25 (G. Terrance Walker et al., *Nucleic Acids Res.* 22, 2670-77 (1994); European Patent Publication Number 0 684 315 entitled *Strand Displacement Amplification Using Thermophilic Enzymes*). In essence, this process involves the following three steps, which altogether comprise a cycle: 1) denaturing a double stranded (ds) DNA molecule containing the sequence to be amplified at an appropriate temperature and for an
30 appropriate period of time to obtain the two single stranded (ss) DNA molecules (the template: sense and antisense strand); 2) contacting the template with at least one primer

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(P), that contains a recognition/cleavage site for a restriction endonuclease (RE) and that hybridizes to at least one ss DNA template at an appropriate temperature and for an appropriate period of time to obtain a primer containing ss DNA template; 3) contacting the primer containing template at an appropriate temperature and for an appropriate period of time with: (i) a complete set of chain elongating nucleotides; (ii) at least one chain terminating nucleotide, (iii) a first DNA polymerase, which has a relatively low affinity towards the chain terminating nucleotide; (iv) a second DNA polymerase, which has a relatively high affinity towards the chain terminating nucleotide; and v) an RE that nicks the primer recognition/cleavage site.

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Steps 1) - 3) can be sequentially performed for an appropriate number of times (cycles) to obtain the desired amount of amplified sequencing ladders. As with the PCR based process, the quantity of the base specifically terminated fragment desired dictates how many cycles are performed. Preferably, less than 50 cycles, more preferably less than about 40 cycles and most preferably about 20 to 30 cycles are performed.

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The amplified sequencing ladders obtained as described above, can be separated and detected and/or quantitated using well established methods, such as polyacrylamide gel electrophoresis (PAGE), or capillary zone electrophoresis (CZE) (Jorgenson *et al.*, *J. Chromatography* 352, 337 (1986); Gesteland *et al.*, *Nucleic Acids Res.* 18, 1415-1419 (1990)); or direct blotting electrophoresis (DBE) (Beck and Pohl, *EMBO J.*, vol. 3: Pp. 2905-2909 (1984)) in conjunction with, for example, colorimetry, fluorimetry, chemiluminescence and radioactivity.

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Dye-terminator chemistry can be employed in the combined amplification and sequencing reaction to enable the simultaneous generation of forward and reverse sequence ladders, which can be separated based on the streptavidin-biotin system when one biotinylated primer is provided.

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Figure 3 depicts a scheme for the combined amplification and sequencing using two polymerases and dye-labeled chain terminating nucleotide (ddNTP) for detection and two reverse oriented primers. A means of separation for the

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simultaneously generated forward and reverse sequence ladders is shown. Step A represents the exponential amplification of a target sequence by the polymerase with a low affinity for ddNTPs. One of the sequence specific oligonucleotide primers is biotinylated. Step B represents the generation of a sequence ladder either from the original template or the simultaneously generated amplification product carried out by the polymerase with a high affinity for ddNTPs. After completion of the reaction, the products are incubated with a streptavidin coated solid support (Step C). Biotinylated forward sequencing products and reverse products hybridized to the forward template are immobilized. In order to obtain readable sequence information, the forward and reverse sequence ladders are separated in Step D. The immobilized strands are washed and separated by denaturation with ammonium hydroxide at room temperature. The non-biotinylated reverse sequencing products are removed from the beads with ammonium hydroxide supernatant during this procedure. The biotinylated forward sequencing products remain immobilized to the beads and are re-solubilized with ammonium hydroxide at 60°C. After ethanol precipitation, both sequencing species can be resuspended in loading dye and run on an automated sequencer, for example.

When mass spectrometry is used in conjunction with the direct amplification and chain termination processes, the sequencing ladders can be directly detected without first being separated using several mass spectrometer formats. Amenable formats for use in the invention include ionization techniques such as matrix-assisted laser desorption (MALDI), continuous or pulsed electrospray (ESI) and related methods (e.g. Ionspray or Thermospray), and massive cluster impact (MSI); these ion sources can be matched with a detection format, such as linear or reflectron time-of-flight (TOF), single or multiple quadrupole, single or multiple magnetic sector, Fourier Transform ion cyclotron resonance (FTICR), ion trap, or combinations of these to give a hybrid detector (e.g. ion trap-TOF). For ionization, numerous matrix/wavelength combinations (MALDI) or solvent combinations (ESI) can be employed.

The above-described process can be performed using virtually any nucleic acid molecule as the source of the DNA template. For example, the nucleic acid

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molecule can be: a) single stranded or double stranded; b) linear or covalently closed circular in supercoiled or relaxed form; or c) RNA if combined with reverse transcription to generate a cDNA. For example, reverse transcription can be performed using a suitable reverse transcriptase (e.g. Moloney murine leukemia virus reverse transcriptase) using standard techniques (e.g. Kawasaki (1990) in *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, Berkeley, CA pp21-27).

Sources of nucleic acid templates can include: a) plasmids (naturally occurring or recombinant); b) RNA- or DNA- viruses and bacteriophages (naturally occurring or recombinant); c) chromosomal or episomal replicating DNA (e.g. from tissue, a blood sample, or a biopsy); d) a nucleic acid fragment (e.g. derived by exonuclease, unspecific endonuclease or restriction endonuclease digestion or by physical disruption (e.g. sonication or nebulization)); and e) RNA or RNA transcripts like mRNAs.

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The nucleic acid to be amplified and sequenced can be obtained from virtually any biological sample. As used herein, the term "biological sample" refers to any material obtained from any living source (e.g. human, animal, plant, bacteria, fungi, protist, virus). Examples of appropriate biological samples for use in the instant invention include: solid materials (e.g. tissue, cell pellets, biopsies) and biological fluids (e.g. urine, blood, saliva, amniotic fluid, mouth wash, spinal fluid). The nucleic acid to be amplified and sequenced can be provided by unpurified whole cells, bacteria or virus. Alternatively, the nucleic acid can first be purified from a sample using standard techniques, such as: a) cesium chloride gradient centrifugation; b) alkaline lysis with or without RNase treatment; c) ion exchange chromatography; d) phenol/chloroform extraction; e) isolation by hybridization to bound oligonucleotides; f) gel electrophoresis and elution; alcohol precipitation and h) combinations of the above.

As used herein, the phrases "chain-elongating nucleotides" and "chain-terminating nucleotides" are used in accordance with their art recognized meaning. For example, for DNA, chain-elongating nucleotides include 2'-deoxyribonucleotides (e.g.

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dATP, dCTP, dGTP and dTTP) and chain-terminating nucleotides include 2', 3'-dideoxyribonucleotides (e.g. ddATP, ddCTP, ddGTP, ddTTP). For RNA, chain-elongating nucleotides include ribonucleotides (e.g., ATP, CTP, GTP and UTP) and chain-terminating nucleotides include 3'-deoxyribonucleotides (e.g. 3'dA, 3'dC, 3'dG and 3'dU). A complete set of chain elongating nucleotides refers to dATP, dCTP, dGTP and dTTP. The term "nucleotide" is also well known in the art. For the purposes of this invention, nucleotides include nucleoside mono-, di-, and triphosphates. Nucleotides also include modified nucleotides such as phosphorothioate nucleotides and deazapurine nucleotides. A complete set of chain-elongating nucleotides refers to four different nucleotides that can hybridize to each of the four different bases comprising the DNA template.

If the amplified sequencing ladders are to be detected by mass spectrometric analysis, it may be useful to "condition" nucleic acid molecules, for example to decrease the laser energy required for volatilization and/or to minimize fragmentation. Conditioning is preferably performed while the sequencing ladders are immobilized. An example of conditioning is modification of the phosphodiester backbone of the nucleic acid molecule (e.g. cation exchange), which can be useful for eliminating peak broadening due to a heterogeneity in the cations bound per nucleotide unit.

Contacting a nucleic acid molecule, which contains an -thio-nucleoside-triphosphate during polymerization with an alkylating agent such as alkyl iodide, iodoacetamide, -iodoethanol, or 2,3-epoxy-1-propanol, the monothio phosphodiester bonds of a nucleic acid molecule can be transformed into a phosphotriester bond. Further conditioning involves incorporating nucleotides which reduce sensitivity for depurination (fragmentation during MS), e.g. a purine analog such as N7- or N9-deazapurine nucleotides, and partial RNA containing oligodeoxynucleotide to be able to remove the unmodified primer from the amplified and modified sequencing ladders by RNase or alkaline treatment. In DNA sequencing using fluorescent detection and gel electrophoretic separation, the N7 deazapurine nucleotides reduce the formation of secondary structure resulting in band compression from which no sequencing information can be generated.

polymerization and yields sequencing products. Preferably about 0.5 to about 3 units of polymerase is used in the combined amplification and chain termination reaction. Most preferably about 1 to 2 units is used. Particularly preferred polymerases for use in conjunction with PCR or other thermal amplification process are thermostable polymerases, such as Taq DNA polymerase (Boehringer Mannheim), AmpliTaq FS DNA polymerase (Perkin-Elmer), Deep Vent (exo⁻), Vent, Vent (exo⁻) and Deep Vent DNA polymerases (New England Biolabs), Thermo Sequenase (Amersham) or exo(-) *Pseudococcus furiosus* (*Pfu*) DNA polymerase (Stratagene, Heidelberg Germany).

15 AmpliTaq, Ultiman, 9 degree Nm, Tth, Hot Tub, and *Pyrococcus furiosus*. In addition, preferably the polymerase does not have 5'-3' exonuclease activity.

As shown in Example 1, the process of the invention can be carried out using AmpliTaq FS DNA polymerase (Perkin-Elmer), which has a relatively high affinity and Taq DNA polymerase, which has a relatively low affinity for chain terminating nucleotides. Other appropriate polymerase pairs for use in the instant invention can be determined by one of skill in the art. (See e.g. S. Tabor and C.C. Richardson (1995) *Proc. Nat. Acad. Sci. (USA)*, vol. 92: Pp. 6339-6343.)

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25 In addition to polymerases, which have a relatively high and a relatively low affinity to the chain terminating nucleotide, a third polymerase, which has proofreading capacity (e.g. *Pyrococcus woesei* (*Pwo*)) DNA polymerase may also be added to the amplification mixture to enhance the fidelity of amplification.

30 Oligonucleotide primers, for use in the invention, can be designed based on knowledge of the 5' and/or 3' regions of the nucleotide sequence to be

antisense strand of the template DNA has been performed.

Examples of appropriate solid supports include beads (silica gel,

10 controlled pore glass, magnetic beads, Sephadex/Sephadex beads, cellulose beads, etc.), capillaries, flat supports such as glass fiber filters, glass surfaces, metal surfaces (steel, gold, silver, aluminum, and copper), plastic materials or membranes (polyethylene, polypropylene, polyamide, polyvinylidene difluoride) or beads in pits of flat surfaces such as wafers (e.g. silicon wafers), with or without filter plates.

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Immobilization can be accomplished, for example, based on

hybridization between a capture nucleic acid sequence, which has already been immobilized to the support and a complementary nucleic acid sequence, which is also contained within the nucleic acid molecule containing the nucleic acid sequence to be detected. So that hybridization between the complementary nucleic acid molecules is not hindered by the support, the capture nucleic acid can include a spacer region of at least about five nucleotides in length between the solid support and the capture nucleic acid sequence. The duplex formed will be cleaved under the influence of the laser

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pulse and desorption can be initiated. The solid support-bound base sequence can be presented through natural oligonucleotide or oligodeoxyribonucleotide as well as analogs (e.g. thio-modified phosphodiester or phosphotriester backbone) or employing oligonucleotide mimetics such as PNA analogs (see e.g. Nielsen *et al.*, *Science*, 254, 1497 (1991)) which render the base sequence less susceptible to enzymatic degradation and hence increases overall stability of the solid support-bound capture

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30 base sequence.

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Alternatively, a target detection site can be directly linked to a solid support via a reversible or irreversible bond between an appropriate functionality (L') on the target nucleic acid molecule and an appropriate functionality (L) on the capture molecule. A reversible linkage can be such that it is cleaved under the conditions of mass spectrometry (i.e., a photocleavable bond such as a trityl ether bond or a charge transfer complex or a labile bond being formed between relatively stable organic radicals). Furthermore, the linkage can be formed with L' being a quaternary ammonium group, in which case, preferably, the surface of the solid support carries negative charges which repel the negatively charged nucleic acid backbone and thus facilitate the desorption required for analysis by a mass spectrometer. Desorption can occur either by the heat created by the laser pulse and/or, depending on L', by specific absorption of laser energy which is in resonance with the L' chromophore.

By way of example, the L-L' chemistry can be of a type of disulfide bond (chemically cleavable, for example, by mercaptoethanol or dithioerythrol), a biotin/streptavidin system, a heterobifunctional derivative of a trityl ether group (Köster *et al.*, "A Versatile Acid-Labile Linker for Modification of Synthetic Biomolecules," *Tetrahedron Letters* **31**, 7095 (1990)) which can be cleaved under mildly acidic conditions as well as under conditions of mass spectrometry, a levulinyl group cleavable under almost neutral conditions with a hydrazinium/acetate buffer, an arginine-arginine or lysine-lysine bond cleavable by an endopeptidase enzyme like trypsin or a pyrophosphate bond cleavable by a pyrophosphatase or a ribonucleotide in between a deoxynucleotide sequence cleavable by an RNase or alkali.

The functionalities, L and L', can also form a charge transfer complex and thereby form the temporary L-L' linkage. Since in many cases the "charge-transfer band" can be determined by UV/vis spectrometry (see e.g. *Organic Charge Transfer Complexes* by R. Foster, Academic Press, 1969), the laser energy can be tuned to the corresponding energy of the charge-transfer wavelength and, thus, a specific desorption off the solid support can be initiated. Those skilled in the art will recognize that several combinations can serve this purpose and that the donor

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functionality can be either on the solid support or coupled to the nucleic acid molecule to be detected or vice versa.

5 In yet another approach, a reversible L-L' linkage can be generated by homolytically forming relatively stable radicals. Under the influence of the laser pulse, desorption (as discussed above) as well as ionization will take place at the radical position. Those skilled in the art will recognize that other organic radicals can be selected and that, in relation to the dissociation energies needed to homolytically cleave the bond between them, a corresponding laser wavelength can be selected (see 10 e.g. Reactive Molecules by C. Wentrup, John Wiley & Sons, 1984).

15 An anchoring function L' can also be incorporated into a target capturing sequence by using appropriate primers during an amplification procedure, such as PCR, LCR or transcription amplification.

For certain applications, it may be useful to simultaneously amplify and chain terminate more than one (mutated) loci on a particular captured nucleic acid fragment (on one spot of an array) or it may be useful to perform parallel processing by using oligonucleotide or oligonucleotide mimetic arrays on various solid supports. 20 "Multiplexing" can be achieved either by the sequence itself (composition or length) or by the introduction of mass-modifying functionalities into the primer oligonucleotide. Such multiplexing is particularly useful in conjunction with mass spectrometric DNA sequencing or mobility modified gel based fluorescence sequencing.

25 Without limiting the scope of the invention, the mass or mobility modification can be introduced by using oligo-/polyethylene glycol derivatives. The oligo/polyethylene glycols can also be monoalkylated by a lower alkyl such as methyl, ethyl, propyl, isopropyl, t-butyl and the like. Other chemistries can be used in the mass-modified compounds, as for example, those described recently in Oligonucleotides and Analogues, A Practical Approach, F. Eckstein, editor IRL Press, Oxford, 1991. 30

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In yet another embodiment, various mass or mobility modifying functionalities, other than oligo/polyethylene glycols, can be selected and attached via appropriate linking chemistries. A simple modification can be achieved by using different alkyl, aryl or aralkyl moieties such as methyl, ethyl, propyl, isopropyl, t-butyl, hexyl, phenyl, substituted phenyl or benzyl. Yet another modification can be obtained by attaching homo- or heteropeptides to the nucleic acid molecule (e.g., primer) or nucleoside triphosphates. Simple oligoamides also can be used. Numerous other possibilities, in addition to those mentioned above, can be performed by one skilled in the art.

10

Different mass or mobility modified primers allow for multiplex sequencing via simultaneous detection of primer-modified Sanger sequencing ladders. Mass or mobility modifications can be incorporated during the amplification process through nucleoside triphosphates or modified primers.

15

For use with certain detection means, such as polyacrylamide gel electrophoresis (PAGE), detectable labels must be used in either the primer (typically at the 5'-end) or in one of the chain extending nucleotides, or chain terminating nucleotides. Using radioisotopes such as ^{32}P , ^{33}P , or ^{35}S is still the most frequently used technique. After PAGE, the gels are exposed to X-ray films and silver grain exposure is analyzed.

20

Non-radioactive labeling techniques have been explored and, in recent years, integrated into partly automated DNA sequencing procedures. All these improvements utilize the Sanger sequencing strategy. The label (e.g. fluorescent dye) can be tagged to the primer (Smith *et al.*, Nature **321**, 674-679 (1986) and EPO Patent No. 87300998.9; Du Pont De Nemours EPO Application No. 0359225; Ansorge *et al.*, J. Biochem. Biophys. Methods **13**, 325-32 (1986)) or to the chain-terminating dideoxynucleoside triphosphates (Prober *et al.*, Science **238**, 336-41 (1987); Applied Biosystems, PCT Application WO 91/05060). Based on either labeling the primer or the ddNTP, systems have been developed by Applied Biosystems (Smith *et al.*, Science **235**, G89 (1987); U.S. Patent Nos. 570973 and 689013), Du Pont De

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Nemours (Prober *et al.* Science 238, 336-341 (1987); U.S. Patents Nos. 881372 and 57566), Pharmacia-LKB (Ansorge *et al.* Nucleic Acids Res. 15, 4593-4602 (1987) and EMBL Patent Application DE P3724442 and P3805808.1) and Hitachi (JP 1-90844 and DE 4011991 A1). A somewhat similar approach was developed by

5 Brumbaugh *et al.* (Proc. Natl. Sci. USA 85, 5610-14 (1988) and U.S. Patent No. 4,729,947). An improved method for the Du Pont system using two electrophoretic lanes with two different specific labels per lane is described (PCT Application WO92/02635). A different approach uses fluorescently labeled avidin and biotin labeled primers. Here, the sequencing ladders ending with biotin are reacted during

10 electrophoresis with the labeled avidin which results in the detection of the individual sequencing bands (Brumbaugh *et al.*, U.S. Patent No. 594676).

More recently even more sensitive non-radioactive labeling techniques for DNA using chemiluminescence triggerable and amplifiable by enzymes have been

15 developed (Beck, O'Keefe, Coull and Köster, Nucleic Acids Res. 17, 5115-5123 (1989) and Beck and Köster, Anal. Chem. 62, 2258-2270 (1990)). These labeling methods were combined with multiplex DNA sequencing (Church *et al.* Science 240, 185-188 (1988) and direct blotting electrophoresis (DBE) (Beck and Pohl, EMBO J., Vol. 3: p 2905-2909 (1984)) to provide for a strategy aimed at high throughput DNA

20 sequencing (Köster *et al.*, Nucleic Acids Res. Symposium Ser. No. 24, 318-321 (1991), University of Utah, PCT Application No. WO 90/15883). However, this strategy still suffers from the disadvantage of being very laborious and difficult to automate.

25 In automated sequencing, fluorescence labeled DNA fragments are detected during migration through the sequencing gel by laser excitation. Fluorescence label is incorporated during the sequencing reaction via labeled primers or chain extending nucleotides (Smith, L. *et al.*, Fluorescence detection in automated DNA sequence analysis, Nature 321:674-89 1986), (Knight, P., Automated DNA

30 sequencers, Biotechnology 6:1095-96 1988).

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Multiple distinctly labeled primers can be used to discriminate sequencing patterns. For example, four differently labeled sequencing primers specific for the single termination reactions, e.g. with fluorescent dyes and online detection using laser excitation in an automated sequencing device. The use of eight differently labeled
5 primers allow the discrimination of the sequencing pattern from both strands. Instead of labeled primers, labeled ddNTP may be used for detection, if separation of the sequencing fragments derived from both strand is provided. With one biotin labeled primer, sequencing fragments from one strand can be isolated for example via biotin-streptavidin coated magnetic beads. Possible is also the isolation via immunoaffinity
10 chromatography in the case of a digoxigenin labeled primer or with affinity chromatography in case of complementary oligonucleotides bound to a solid support.

Another aspect of this invention concerns kits for directly generating from a nucleic acid template, amplified base specifically terminated fragments. Such
15 kits include combinations of the above-described reactants. For instance, in one embodiment, the kit can comprise: i) a set of chain-elongating nucleotides; ii) a set of chain-terminating nucleotides; and (iii) a first DNA polymerase, which has a relatively low affinity towards the chain terminating nucleotide; and (iv) a second DNA
20 polymerase, which has a relatively high affinity towards the chain terminating nucleotide. The kit can also include appropriate solid supports for capture/purification and buffers as well as instructions for use.

The present invention is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited
25 references (including literature references, issued patents, published patent applications (including international patent application Publication Number WO 94/16101, entitled *DNA Sequencing by Mass Spectrometry* by H. Koester; and international patent application Publication Number WO 94/21822 entitled "DNA Sequencing by Mass Spectrometry Via Exonuclease Degradation" by H. Köster), and
30 co-pending patent applications, (including U.S Patent Application Serial No. 08/406,199, entitled *DNA Diagnostics Based on Mass Spectrometry* by H. Koester),

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and continuations-in-part of co-pending patent applications (published or unpublished) are hereby expressly incorporated by reference.

Example 1: Direct Nucleic Acid Amplification and Sequencing

Materials and Methods

Bacterial Strains and Plasmids

E. coli K12 strains HB101 and XL1-Blue (Stratagene, California) were grown in LB broth (Miller, J. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.) supplemented with 100 µg ampicillin/mL (Binotal, Bayer, Germany). DNA-template used for sequencing was a pOM8 derived recombinant plasmid (Oberbäumer, I. (1986) Gene 49: 81-91 1986) with an 400 BP insert from the *rrnB* gene of *E. coli*. The plasmid DNA was purified via Diagen plasmid purification tips-100 (Diagen, Hilden, Germany).

DNA Isolation

The DNA template used in the sequencing reaction was isolated according to the method described by Birnboim and Doly (Birnboim, H.C., and Doly, J. Nucleic Acid Res. 7: 1513-1523 1979) with the improvements of Ish-Horowicz and Burke (Ish-Horowicz, D., and Burke, J.F. Nucleic Acid Res. 9: 2989-2998 1981).

Sequencing reactions

A control sequencing reaction was performed in accordance with the Perkin-Elmer, AmpliTaq FS kit protocol.

A combined nucleic acid amplification and sequencing reaction was performed as follows. The base specific reaction mixtures (A, C, G and T, as described below) each contained a buffered solution of: i) DNA template; ii) two thermostable DNA polymerases, each with a different affinity to the ddNTP; iii) the ddNTP; iv) all 4 dNTPs (Pharmacia, Freiburg, Germany); a pair of sequence specific oligonucleotide primers, one of them labeled with a fluorescent dye. The reaction mixture was overlaid with wax.

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The sequencing reaction was carried out as follows:

5 A-reaction: 1.8 M ddATP, 80 M each dATP, dCTP, 7-deaza-dGTP, dTTP, 500 mM Tris-HCl (pH 9.0 at 25°C), 25 mM MgCl₂ containing 1 pmol JOE dye primer, thermal stable pyrophosphatase and 1.6 U AmpliTaq DNA polymerase, FS. To this 50 ng of the DNA template as prepared above (1 l), 1 U of Taq DNA polymerase (1 l) and 10 pmol reverse primer (0.5 l) were pipetted.

10 C-reaction: 4.18 M ddCTP, 80 M each dATP, dCTP, 7-deaza-dGTP, dTTP, 500 mM Tris-HCl (pH 9.0 at 25°C), 25 mM MgCl₂ containing 1 pmol FAM dye primer, thermal stable pyrophosphatase and 1.6 U AmpliTaq DNA polymerase, FS. To this 50 ng of the DNA template (1 l), 1 U of Taq DNA polymerase (1 l) and 10 pmol reverse primer (0.5 l) were pipetted.

15 G-reaction: 8.18 M ddGTP, 80 M each dATP, dCTP, 7-deaza-dGTP, dTTP, 500 mM Tris-HCl (pH 9.0 at 25°C) 25mM MgCl₂ containing 1 pmol TAMRA dye primer, thermal stable pyrophosphatase and 1.6 U AmpliTaq DNA polymerase, FS. To this 50 ng of the DNA template (1 l), 1 U of Taq DNA polymerase (1 l) and 10 pmol reverse primer (0.5 l) were pipetted.

25 T-reaction: 8.18 M ddTTP, 80 M each dATP, dCTP, 7-deaza-dGTP, dTTP, 500 mM Tris-HCl (pH 9.0 at 25°C), 25mM MgCl₂ containing 1 pmol ROX dye primer, thermal stable pyrophosphatase and 1.6 U AmpliTaq DNA polymerase, FS. To this 50 ng of the DNA template (1 l), 1 U of Taq DNA polymerase (1 l) and 10 pmol reverse primer (0.5 l) were pipetted.

30 The incubation conditions included an initial denaturation step of 4 min. 95°C, followed by 15 cycles of 30 sec. 95°C, 30 sec. 52°C, 60 sec. 72°C. The reaction is completed by additional 15 cycles of 30 sec. 95°C, 30 sec. 52°C, 60 sec. 72°C.

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The reaction mixture was separated from the wax by pipetting and ethanol precipitated. The samples were run on an automated ABI prism sequencer model 377.

5 **RESULTS**

As can be seen from Figures 1 and 2, while the combined amplification and sequencing reaction yielded a 440 BP readable sequence with 15 ng. of template DNA (Figure 1), no sequence data was obtained employing the same amount of template
10 DNA and using the standard cycle sequencing protocol (Figure 2).

Example 2: Direct Nucleic Acid Amplification and Sequencing

A combined nucleic acid amplification and sequencing reaction was performed as follows. The reaction contained a buffered solution of i) DNA template,
15 ii) two thermostable DNA polymerases, each with a different affinity for the ddNTP; iii) dye-labeled ddNTP; iv) all 4 dNTP s (Pharmacia, Freiburg, Germany); a pair of sequence specific oligonucleotide primers, one of them labeled with a biotin group.

Combined amplification and sequencing protocol using two DNA 20 polymerases.

4ng (1 µl) of p9G551D (pUC-derivative harbouring a 419 BP insert from human mutant CFTR gene) was subjected to sequencing by adding 4 µl of 5 x TACS buffer, 0.5 µl of each dye ddNTP solution, 1 µl dNTP mix (5mM dATP, dCTP, dTTP and 10 mM N⁷ deaza dGTP), 1 µl AmpliTaq FS, 0.75 U (0.3 µl) (exo-) *Pfu* polymerase
25 and 40 pmol of each of the standard M13 primers (for sequence see above) to a final volume of 20 µl. The cycling profile comprised one cycle of 95°C for 4 min., 20 cycles of 95°C for 45 sec, 52°C for 45 sec and 72°C for 45 sec. followed by 28 cycles of 96°C for 50 sec, 55°C for 60 sec and 60°C for 4 min. The reaction was carried out using an Eppendorf Mastercycler 5330 with a heated lid.
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Separation of the forward and reverse sequencing fragments.

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The reaction was mixed with an equal volume of 2x B/W-buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA, 2 M NaCl) containing 300 µg of prewashed streptavidin M-280 Dynabeads (Dynal). After incubation for 30 min. at room temperature the beads were washed twice with 50 µl of 1x B/W buffer in order to remove unwanted reaction components and unincorporated dye terminators.

The reverse sequencing products were recovered from immobilized biotinylated forward products by addition of 20 µl of a 20% aqueous ammonium hydroxide solution (freshly prepared) and incubation at room temperature for two minutes. This step was repeated once the products were pooled.

The beads were then washed with 20 µl 25% aqueous ammonium hydroxide solution for one minute at room temperature and the supernatant was discarded.

The forward sequencing reaction was removed from the beads using 30 µl of a 25% aqueous ammonium hydroxide solution and incubation at 60°C for 8 minutes. This step was also repeated once and the products were pooled.

The separated forward and reverse sequencing reactions were finally precipitated each by addition of 200 µl ethanol to the ammonium hydroxide solution, resuspended in 4 µl loading dye (5:1 ratio of deionized formamide to 25mM EDTA (pH 8.0) /50 mg/ml Blue dextran) and analyzed with an ABI Prism sequencer (model 377).

25

RESULTS

Figure 4 and 5 depict the fluorescent chromatogram plots of a forward (Fig. 4) and reverse (Fig. 5) sequence ladder generated in a simultaneous amplification and sequencing reaction. In this experiment 1.5 units of *Taq* polymerase were added to a dye terminator cycle sequencing mix containing AmpliTaq FS, and only 4 ng of plasmid DNA, a biotinylated forward primer and a reverse primer. After the cycling reaction,

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biotinylated forward sequencing products and reverse products hybridized to the forward template were immobilized on streptavidin coated magnetic beads. A separation of reverse and forward sequencing ladders was achieved by incubation of the beads with ammonium hydroxide. Ammonium hydroxide acted as a denaturing agent for double stranded DNA at room temperature, therefore releasing the reverse sequencing products in the solution. The biotinylated products remained bound to the streptavidin, whereas the reverse products were removed with the supernatant. A recovery of the biotinylated products was carried out using ammonium hydroxide at an incubation temperature of 60°C. At elevated temperature, ammonium hydroxide acted as a denaturing agent for the streptavidin biotin interaction. The thus separated sequencing ladders were then ethanol precipitated and separately analyzed on a ABI Prism sequencer.

Equivalents

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation numerous equivalents to the specific procedures

5 described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

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Claims

1. A kit for obtaining amplified and chain terminated nucleic acid molecules from a nucleic acid template comprising: i) an appropriate amount of a set of chain-
5 elongating nucleotides; ii) an appropriate amount of at least one chain-terminating nucleotide; iii) an appropriate amount of a first polymerase, which has a relatively low affinity towards at least one chain terminating nucleotide; and iv) an appropriate amount of a second polymerase, which has a relatively high affinity towards at least one chain
10 terminating nucleotide.
2. A kit of claim 1, which additionally comprises a restriction enzyme that can cleave the primer.
3. A kit of claim 1, wherein the first and second polymerases are
15 thermostable DNA polymerases.
4. A kit of claim 3, wherein the thermostable DNA polymerases are selected from the group consisting of: Taq DNA polymerase, AmpliTaq FS DNA polymerase, Deep Vent (exo⁻) DNA polymerase, Vent DNA polymerase, Vent (exo⁻) DNA
20 polymerase and Deep Vent DNA polymerases, Thermo Sequenase, exo(-) *Pseudococcus furiosus* (Pfu) DNA polymerase, AmpliTaq, Ultman, 9 degree Nm, Tth, Hot Tub, *Pyrococcus furiosus* (Pfu), and *Pyrococcus woesei* (Pwo) DNA polymerase.
5. A kit of claim 1, wherein the set of chain-elongating nucleotides are
25 selected from the group consisting of: i) at least one deoxyadenosine triphosphate; ii) at least one deoxyguanosine triphosphate; iii) at least one deoxycytidine triphosphate; and iv) at least one thymidine triphosphate.
6. A kit of claim 5, wherein the deoxyadenosine and/or the deoxyguanosine
30 is an N7- or N9- deazapurine nucleotide.

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7. A kit of claim 1, wherein the chain terminating nucleotide is selected from the group consisting of: 2',3'-dideoxyadenosine triphosphate, 2',3'-dideoxyguanosine triphosphate, 2',3'-dideoxycytidine triphosphate, 2',3'-dideoxythymidine triphosphate.
- 5 8. A kit of claim 1, which additionally includes at least one primer.
9. A kit of claim 8, wherein the primer is linked to a solid support.
- 10 10. A kit of claim 9, wherein the solid support is selected from the group consisting of beads, capillaries, flat supports, membranes and wafers.
11. A kit of claim 9, wherein the primer contains a restriction site or a ribonucleotide.
- 15 12. A kit of claim 8, wherein the primer is weight modified.
13. A kit of claim 8, wherein the primer is mobility modified.
14. A kit of claim 7, wherein at least one ddNTP is weight modified.
- 20 15. A kit of claim 1, which additionally comprises a proof reading DNA polymerase.
16. A kit of claim 1, which additionally comprises a reverse transcriptase.
- 25 17. A process for simultaneously amplifying and sequencing a single stranded nucleic acid molecule, comprising the steps of:
- 30 a) contacting the single stranded nucleic acid molecule with: i) at least one primer that can hybridize to the template, (ii) a set of chain

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- elongating nucleotides, (iii) at least one chain terminating nucleotide,
(iv) a first DNA polymerase, which has a relatively low affinity towards
the chain terminating nucleotide; and (v) a second DNA polymerase,
which has a relatively high affinity towards the chain terminating
- 5 nucleotide, so that polymerization by the first polymerase results in
amplification and polymerization by the second polymerase results in the
formation of chain terminated fragments;
- b) detecting the chain terminated fragments by an appropriate detection
10 means; and
- c) aligning the fragments by mobility or molecular weight to determine the
sequence of the DNA template.
- 15 18. A process of claim 17, wherein the first and second polymerases are
thermostable DNA polymerases.
19. A process of claim 12, wherein the set of chain-elongating nucleotides are
selected from the group consisting of: i) at least one deoxyadenosine triphosphate; ii) at
20 least one deoxyguanosine triphosphate; iii) at least one deoxycytidine triphosphate; and
iv) at least one thymidine triphosphate.
20. A process of claim 19, wherein the deoxyadenosine and/or the
deoxyguanosine is an N7- or N9- deazapurine nucleotide.
- 25 21. A process of claim 17, wherein the chain terminating nucleotide is selected
from the group consisting of: 2',3'-dideoxyadenosine triphosphate, 2',3'-dideoxyguanosine
triphosphate, 2',3'-dideoxycytidine triphosphate, 2',3'-dideoxythymidine triphosphate.
- 30 22. A process of claim 17, wherein the detection means is a separation means
selected from the group consisting of: polyacrylamide gel electrophoresis, capillary zone

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electrophoresis and mass spectrometry in conjunction with a visualization means selected from the group consisting of: colorimetry, fluorimetry, chemiluminescence, radioactivity and mass spectrometry.

5 23. A process of claim 17, wherein the detection means is mass spectrometry.

24. A process of claim 23, wherein the mass spectrometry is performed
using an ion source selected from the group consisting of: matrix assisted laser
desorption ionization (MALDI), electrospray (ES), ionspray, thermospray and
10 massive cluster impact; and the detection format is selected from the group consisting
of: time-of-flight; quadrupole, magnetic sector, Fourier transform ion cyclotron
resonance and ion trap.

25. A process of claim 17, wherein the double stranded DNA molecule has
15 been synthesized from RNA using a reverse transcriptase.

26. A process of claim 17, wherein the primer is linked to a solid support.

27. A process of claim 26, wherein the solid support is selected from the
20 group consisting of: beads, capillaries, flat supports, membranes and wafers.

28. A process of claim 26, wherein the primer contains a restriction site or a
ribonucleotide.

25 29. A process of claim 26, wherein the primer is weight modified.

30. A process of claim 26, wherein the primer is mobility modified.

31. A process of claim 26, wherein at least one ddNTP is weight modified.

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32. A process of claim 17, which additionally comprises a proofreading DNA polymerase.

33. A process for simultaneously amplifying and sequencing a nucleic acid molecule, comprising the steps of:

a) denaturing a double stranded DNA molecule at an appropriate temperature and for an appropriate period of time to obtain two complementary single stranded DNA molecules;

b) contacting at least one of the single stranded DNA molecules with a complementary primer at an appropriate temperature and for an appropriate period of time to obtain at least one primer containing single stranded DNA molecule;

c) contacting the at least one primer containing single stranded DNA molecule at an appropriate temperature and for an appropriate period of time with: (i) a set of chain elongating nucleotides, (ii) at least one chain terminating nucleotide, (iii) a first DNA polymerase, which has a relatively low affinity towards the chain terminating nucleotide; and (iv) a second DNA polymerase, which has a relatively high affinity towards the chain terminating nucleotide, so that polymerization by the first polymerase results in amplification and polymerization by the second polymerase results in the formation of chain terminated fragments;

d) repeating steps a) through c) for an appropriate number of times to obtain a sufficient amount of chain terminated fragments for detection; and

e) detecting the chain terminated fragments by an appropriate detection means and aligning the fragments by molecular weight to determine the

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sequence of the DNA template.

34. A process of claim 33, wherein the first and second polymerases are thermostable DNA polymerases.

35. A process of claim 33, wherein the set of chain-elongating nucleotides are selected from the group consisting of: i) at least one deoxyadenosine triphosphate; ii) at least one deoxyguanosine triphosphate; iii) at least one deoxycytidine triphosphate; and iv) at least one thymidine triphosphate.

36. A process of claim 35, wherein the deoxyadenosine and/or the deoxyguanosine is an N7- or N9- deazapurine nucleotide.

37. A process of claim 33, wherein the chain terminating nucleotide is selected from the group consisting of: 2',3'-dideoxyadenosine triphosphate, 2',3'-dideoxyguanosine triphosphate, 2',3'-dideoxycytidine triphosphate, 2',3'-dideoxythymidine triphosphate.

38. A process of claim 33, wherein the detection means is a separation means selected from the group consisting of: polyacrylamide gel electrophoresis, capillary zone electrophoresis and mass spectrometry, in conjunction with a visualization means selected from the group consisting of: colorimetry, fluorimetry, chemiluminescence, radioactivity, and mass spectrometry.

39. A process of claim 33, wherein the detection means is mass spectrometry.

40. A process of claim 39, wherein the mass spectrometry is performed using an ion source selected from the group consisting of: matrix assisted laser desorption ionization (MALDI), electrospray (ES), ionspray, thermospray and massive cluster impact; and the detection format is selected from the group consisting of: time-of-flight; quadrupole, magnetic sector, Fourier transform ion cyclotron resonance and ion trap.

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41. A process of claim 33, wherein the double stranded DNA molecule has been synthesized from RNA using a reverse transcriptase.
- 5 42. A process of claim 33, wherein the primer is linked to a solid support.
43. A process of claim 42, wherein the solid support is selected from the group consisting of beads, capillaries, flat supports, membranes and wafers.
- 10 44. A process of claim 33, wherein the primer contains a restriction or a ribonucleotide.
45. A process of claim 33, wherein the primer is weight modified.
- 15 46. A process of claim 33, wherein the primer is mobility modified.
47. A process of claim 33, wherein a ddNTP is weight modified.
48. A process of claim 33, which additionally comprises a proofreading DNA
20 polymerase.

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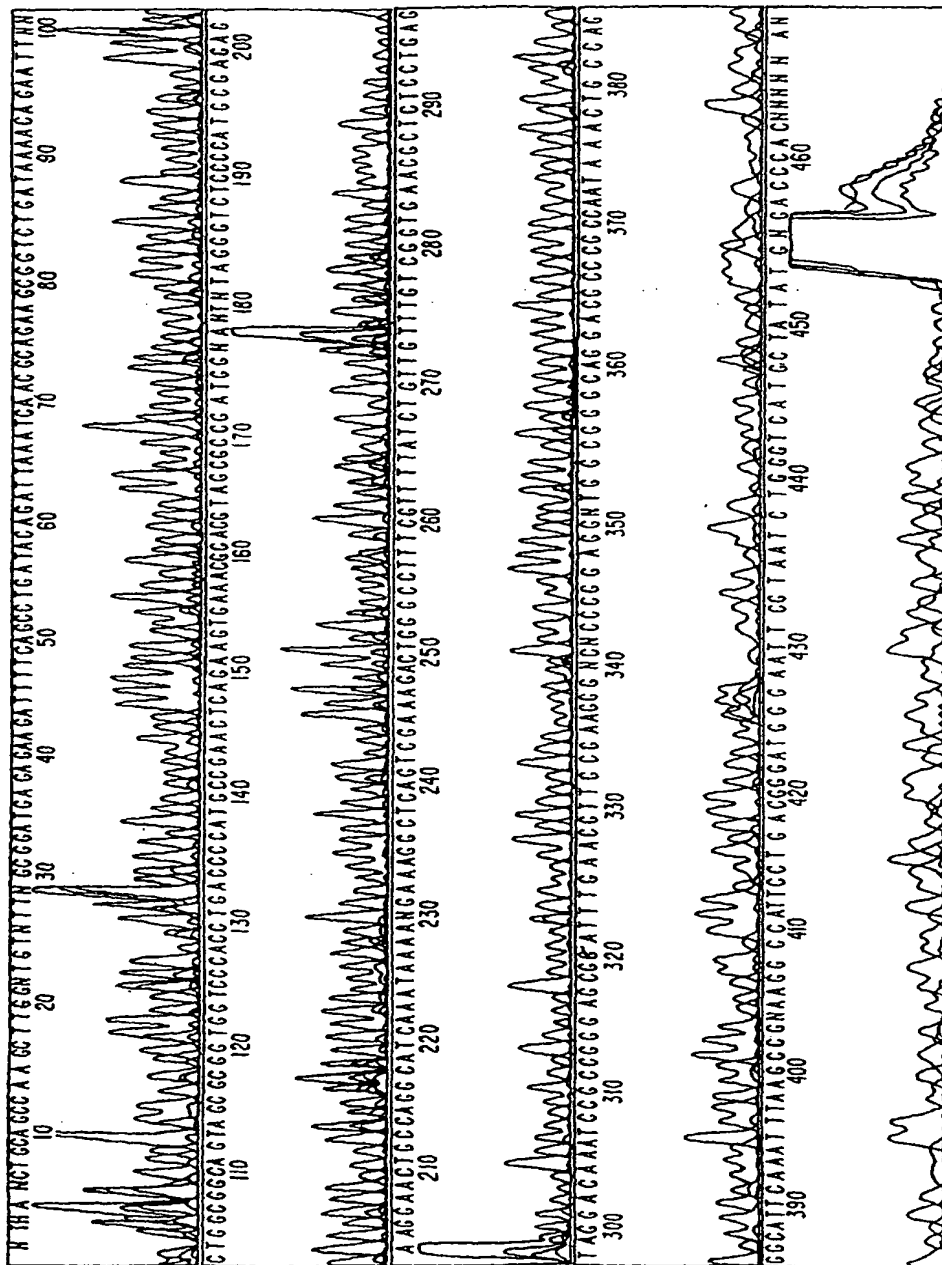
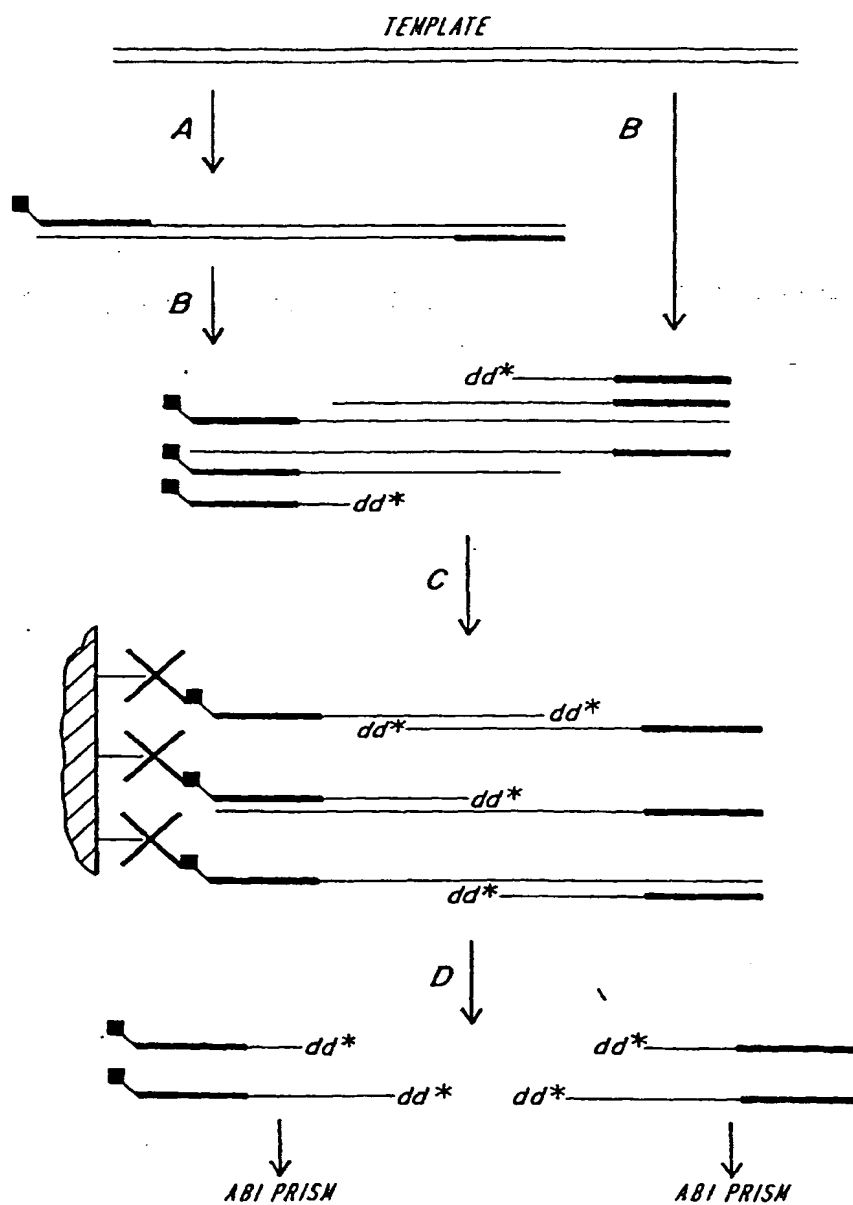


FIG. 1

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**FIG. 3**

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4/5

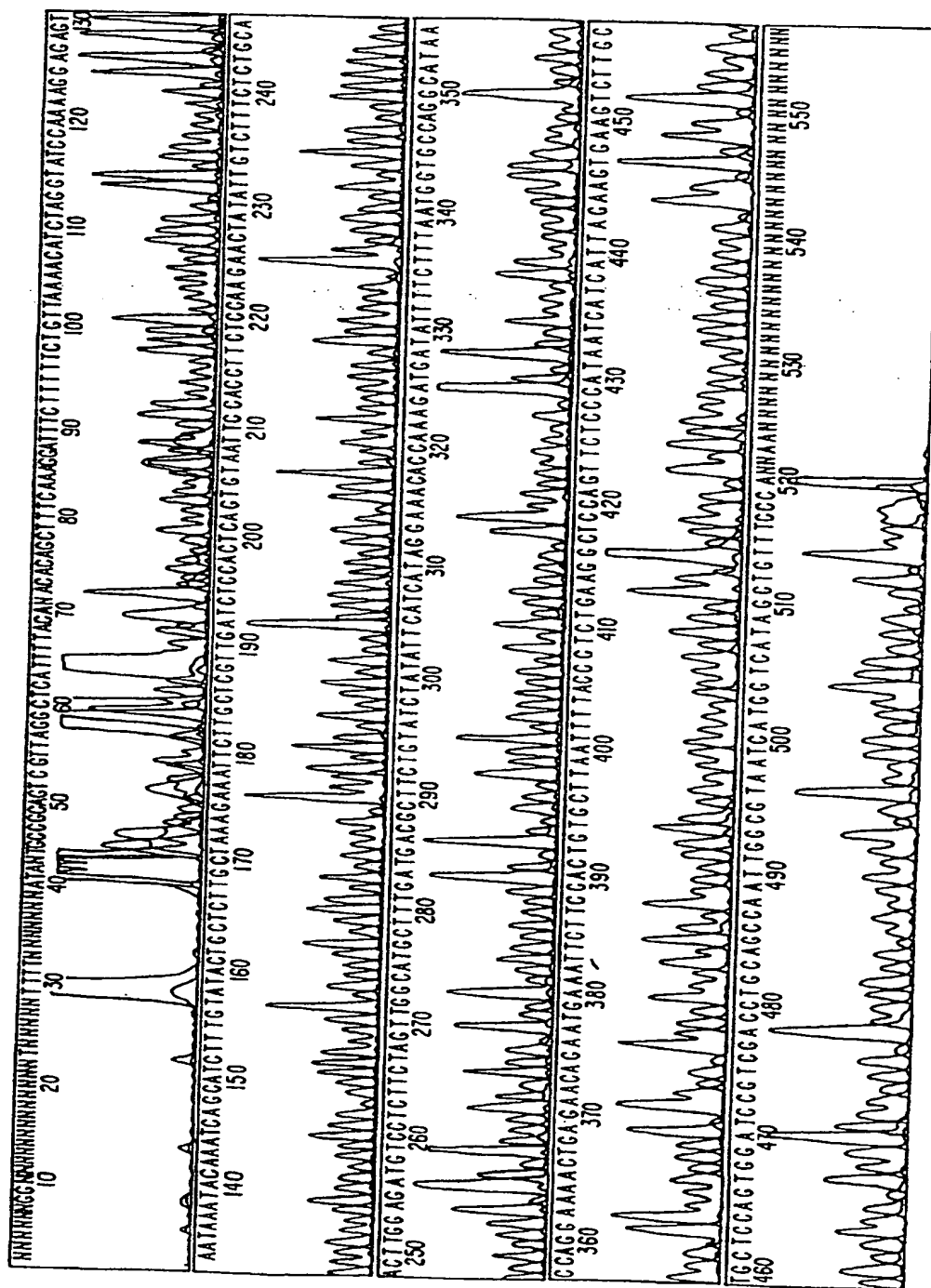


FIG. 4

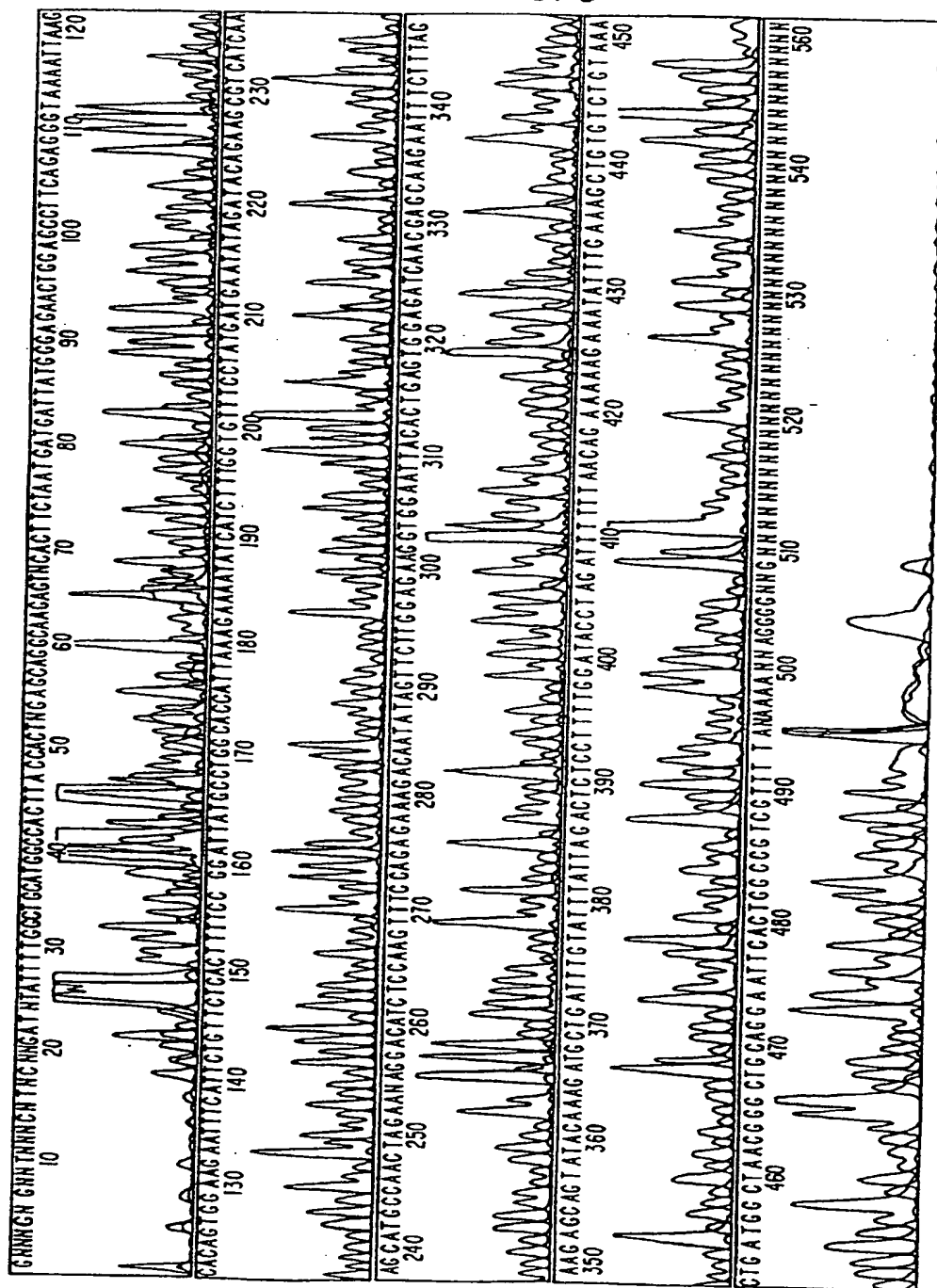


FIG. 5

INTERNATIONAL SEARCH REPORT

Int. Nat. Application No.

PCT/US 97/07966

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	REDSTONE M ET AL: "Klenow Co-Sequencing: A method for eliminating "stops" BIOTECHNIQUES, vol. 17, no. 2, 1994, page 286,288 XP002041095 see the whole document	1,17,33
A	MURRAY V: "IMPROVED DOUBLE-STRANDED DNA SEQUENCING USING THE LINEAR POLYMERASE CHAIN REACTION" NUCLEIC ACIDS RESEARCH, vol. 17, no. 21, 11 November 1989, page 8889 XP000371763 see the whole document	1-47
A	WO 93 02212 A (UNIV PARTNERSHIPS PTY LTD) 4 February 1993 see the whole document	1-47

-/-

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "O" document referring to an oral disclosure, use, exhibition or other means
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Date of the actual completion of the international search

19 September 1997

Date of mailing of the international search report

22.10.97

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/07966

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 427 911 A (RUANO GUALBERTO) 27 June 1995 see the whole document ---	1-47
A	WO 94 16101 A (KOESTER HUBERT) 21 July 1994 see the whole document ---	1-47
A	WO 93 06243 A (US BIOCHEMICAL CORP) 1 April 1993 see the whole document ---	1,17,43
A	SCHNEIDER K ET AL: "increased stability of nucleic acids containing 7-deaza guanosine and 7-deaza-adenosine may enable rapid DNA sequencing by matrix assisted laser desorption mass spectrometry" NUCLEIC ACIDS RESEARCH, vol. 23, no. 9, May 1995, pages 1570-75, XP000601618 see the whole document -----	6,20,36

2.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/07966

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9302212 A	04-02-93	AU 660484 B	29-06-95
		CA 2114124 A	04-02-93
		EP 0595982 A	11-05-94
		JP 7500004 T	05-01-95
US 5427911 A	27-06-95	NONE	
WO 9416101 A	21-07-94	AU 5992994 A	15-08-94
		CA 2153387 A	21-07-94
		EP 0679196 A	02-11-95
		JP 8509857 T	22-10-96
		US 5547835 A	20-08-96
		US 5605798 A	25-02-97
WO 9306243 A	01-04-93	AU 2573992 A	27-04-93
		CA 2119140 A	01-04-93
		EP 0654091 A	24-05-95
		JP 6510433 T	24-11-94

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